

**PATENT ABSTRACTS OF JAPAN**

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**(54) LIVE CELL PRESERVATIVE**

(57)Abstract:

PURPOSE: To obtain an inexpensive live cell preservative, containing kestose as an active ingredient, having excellent preserving effects and exhibiting effects on not only freezing preservation but also preservation at a low temperature so as not to freeze the live cells.

CONSTITUTION: A live cell preservative containing 1-kestose as an active ingredient, preferably at 0.1-20% (wt./vol.) concentration. The 1-kestose is obtained by reacting a fructosyltransferase produced by *Scopulariopsis brevicaulis* with, e.g. sucrose.

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## CLAIMS

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[Claim(s)]

[Claim 1] The viable cell preservative characterized by containing 1-kestose as an active principle.

[Claim 2] The viable cell preservative according to claim 1 characterized by adjusting 1-kestose concentration to 0.1 - 20% (W/V).

[Claim 3] The viable cell preservative according to claim 1 or 2 characterized by using for preservation of the viable cell chosen from the sperm of mammalian, the sperm of fishes, or the animal culture tissue.

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## DETAILED DESCRIPTION

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[Detailed Description of the Invention]

[0001]

[Industrial Application] In case this invention saves a viable cell at a freezing condition or low temperature for a long period of time, it relates to the sperm of a required viable cell preservative especially the mammals, and fishes, and the viable cell preservative of an animal culture tissue.

[0002]

[Description of the Prior Art] Although the viable cell has a living function and biological activity, and is various and there is utility value, under the conditions that temperature is high, change, a living function, and biological activity will fall thru/or disappear with time because of a metabolic turnover or denaturation. Many researches are done till today, such as JP,60-29471,B which was considered that cryopreservation is suitable for the mothball of such a viable cell, for example, planned the having-two-incomes effectiveness of an intracellular frost damage protector and an extracellular frost damage protector, JP,63-216476,A which used methyl cellulose as a cryoprotective agent, and JP,2-422,A which used the betaine as a cryoprotective agent.

[0003] Generally, the following item is mentioned as conditions for the matter which show the frost damage prevention effectiveness over a viable cell.

\*\* It is the neutral matter (neutral solute).

\*\* It is the low-molecular matter (namely, matter with the high permeability over a cell).

\*\* Have colligative property (it is easy to make colligative property, i.e., hydrogen bond, and the eutectic point is a low property).

[0004] \*\* The high concentration of the toxicity over a cell is also low.

A glycerol and DMSO (dimethylsulfoxide) are in the matter which fulfills these conditions and is now used widely. However, it is rare to use it independently about a glycerol, and the mixed liquor of a glycerol and sugar is widely used for the sperm cryopreservation diluent of an animal, for example, a cow.

[0005] It is raised that that one-time dilution is possible for the merit at a room temperature, a glycerol balancing time's being shortened, and the freezing velocity of the large range are applicable etc.

Moreover, since the frost damage prevention effectiveness of a saccharide excels 5 and 6 \*\*\*\*

(Pentose, Hexose) in 2 and 3 saccharide, the raffinose which are mainly three saccharides has been used conventionally.

[0006]

[Problem(s) to be Solved by the Invention] However, the chemosynthesis of a raffinose is impossible, and if it does not extract and refine, the vegetation, for example, the beat etc., etc. which exists naturally, it cannot come to hand. Since the amount of supply was restricted, it was expensive. Moreover, since DMSO was an organic solvent, actuation of washing in cold water etc. might be needed, generally handling was complicated and what changes to this was desired strongly.

[0007] These people acquired the knowledge of it having already applied for the approach of manufacturing 1-kestose of a high grade cheaply by using cane sugar as a raw material (Japanese Patent Application No. No. 224312 [ two to ]), and the preservation effectiveness of a viable cell being in this 1-kestose. Then, this invention aims at offering the viable cell preservative which used 1-kestose.

[0008]

[Means for Solving the Problem] In order to solve said trouble, let this invention be the viable cell preservative characterized by containing 1-kestose as an active principle. Moreover, let this invention be the viable cell preservative characterized by adjusting 1-kestose concentration to 0.1 - 20% (W/V).

[0009] Moreover, let this invention be the viable cell preservative characterized by using 1-kestose for preservation of the viable cell chosen from the sperm of mammalian, the sperm of fishes, or the animal culture tissue. 1-kestose is so-called kind of a fructo oligosaccharide, fructose carries out 1 molecular binding of the structure to the fructose part of the shoe cloth (GF) which a glucose (G) and fructose (F) combined by beta-2 and 1 association, and it is O-alpha-D-glucopyranosyl. -(1->2)- It is an O-beta-D-hula KUTOFU llano sill. -(1->2)- It is O-beta-D-FURAKUTO furanoside. The molecular formula is C<sub>18</sub>H<sub>32</sub>O<sub>16</sub>, and the molecular weight is 504. Next, the structure expression of 1-kestose is shown.

[0010]

[Formula 1]

[0011] Although the application of the conventional fructo oligosaccharide had the diuretic currently indicated by the BIFIZUSU activity currently indicated by JP,59-53834,B and JP,59-110621,A, all were the mixture of the mixture of a fructo oligosaccharide, i.e., 1 kestose, (GF2), nistose (GF3), and FURAKUTO furanosyl nistose (GF4). The manufacturing method of 1-kestose is indicated by JP,2-163093,A and JP,2-249493,A, and can be acquired by making the cell tosyl transferase which the Scopulariopsis BUREBI cow squirrel (Scopulariopsis brevicaulis) produces act on cane sugar. Moreover, these people have already applied for the approach of manufacturing 1-kestose of a high grade cheaply by using cane sugar as a raw material, as Japanese Patent Application No. No. 224312 [ two to ] .

[0012] According to this producing method, moreover, 1-kestose is industrially obtained in large quantities by the high grade (99.9%) individually. This high grade 1-kestose acquires the knowledge of having the effectiveness which was excellent as a viable cell preservative, and came to complete this invention. Using 1-kestose used in this invention by the concentration suitable for the viable cell to freeze, the concentration is 0.1 - 20% (W/V) of within the limits. Moreover, 1-kestose of this invention can be used as a part or all of a presentation of a viable cell preservative. For example, in the cryopreservation of the sperm of mammalian, use can be carried out 0.1 to 0.5% (W/V) as an alternative of the raffinose which is one constituent in the existing viable cell preservative (a sugar content glycerol and albumen suspension).

[0013] Moreover, in the sperm and animal culture tissue of fishes, 1-kestose can be adjusted to 3 - 20% (W/V), and it can be independently used as a cryopreservation agent as it is. Although it has an example in below and this invention is explained to it, this is instantiation to the last and this invention is not limited to this.

[0014]

[Example 1] About the cryopreservation of the sperm of a cow, the viable cell preservative containing

1-kestose of this invention was used, and it experimented in the preservation effectiveness. The viable cell preservative which consists of a constituent containing the raffinose which is the viable cell preservative currently used from the former as contrast was used. About the viable cell preservative, the raffinose only replaced the thing of contrast and this invention with 1-kestose, and all other components are the same.

[0015] The presentation of the cryopreservation liquid used for the experiment is shown in the next table 1.

[0016]

[Table 1]

[0017] After the experiment conditions of the cryopreservation of the sperm of a cow are -196 degrees C and saved freezing temperature for six months, they were thawed and investigated motility of sperm. The judgment of the vital force of a sperm should observe the sperm under the microscope, should move what moves forward with sufficient vigor (+++), the thing (++) which moves forward, and the head (+), and made (+++) the figure (%) of motility of sperm. The result is shown in the next table 2.

[0018]

[Table 2]

[0019] According to Table 2, it turns out that the viable cell preservative containing 1-kestose of this invention shows effectiveness equivalent to the viable cell preservative containing the conventional raffinose.

[0020]

[Example 2] 1-kestose, the water solution of a raffinose, or the DMSO solution (what was dissolved in 25mM Tris-HCl and pH7.5) was 80microl Added to 20micro of sperms l of a cherry salmon (*Oncorhynchus masou*), and 60microM and a DMSO solution prepared each stock solution, as 1-kestose and a raffinose became 2% about the last concentration. In addition, 1-kestose used the thing of 99.9% of purity. Thus, it put on the hollow with a diameter of 5mm which made 50micro of each prepared stock solution l with the drill on dry ice, and it quick-froze and the frozen sperm pellet was created.

[0021] This sperm pellet was saved at -70 degrees C, it thawed at 4 degrees C two days and 51 days after, and maneuverability was investigated under the microscope. Maneuverability just behind \*\*\*\* (time amount from movement initiation to a halt) was set to 100. The result is shown in the next table 3.

[0022]

[Table 3]

[0023] Table 3 shows having the preservation effectiveness excellent in the viable cell preservative which carried out independent use of the 1-kestose of this invention.

[0024]

[Example 3] After cultivating until it forms a monolayer at 37 degrees C by L-15 culture medium which added fetal calf serum 5%, using the Hela cell of the gun cell origin as a cultured cell, the Hela cell attached to the bottom of a flask is removed by digesting the Hela cell in which the monolayer was formed, by the trypsin. It isolated preparatively with the pipet which sterilized this and suspended in L-15 culture medium which is a general culture medium for animal cell culture so that it may be set to  $3 \times 10^5$  / ml. 1-kestose of 1/5 amount, a glucose, shoe cloth, and DMSO were added to this so that the last concentration might become at 10%. In addition, 1-kestose used the thing of 99.9% of purity. After carrying out the room temperature defrosting of this after preservation for 16 days at 4 degrees C, -20 degrees C, and -80 degrees C and carrying out trypan blue dyeing of the viable count, the number of viable cells was measured by the counting chamber. In addition, if this staining technique is used, the

nucleus of a viable cell will be dyed, but since the nucleus of a dead cell is not dyed, the survival rate after cryopreservation is known. The result is shown in the next table 4.

[0025]

[Table 4]

[0026] It turns out that the viable cell preservative which carried out independent use of the 1-kestose of this invention from Table 4 shows the preservation effectiveness which was excellent also in any (4 degrees C, -20 degrees C, and -80 degrees C).

[0027]

[Example 4] The Vero cell which formed the monolayer after culture at 37 degrees C was digested by the trypsin in L-15 culture medium which added fetal calf serum 5%, using the Vero cell of the nephrocyte of an African green monkey as a cultured cell, and it suspended in L-15 culture medium so that it might be set to  $1.5 \times 10^5$  / ml. 1-kestose of 1/5 amount, a glucose, shoe cloth, and DMSO were added to this so that the last concentration might become at 10%. In addition, 1-kestose used the thing of 99.9% of purity. After carrying out the room temperature defrosting of this after preservation for 16 days at 4 degrees C, -20 degrees C, and -80 degrees C and carrying out trypan blue dyeing of the viable count, it measured by the counting chamber. The result is shown in the next table 5.

[0028]

[Table 5]

[0029] It turns out that the viable cell preservative which carried out independent use of the 1-kestose of this invention from Table 5 shows the preservation effectiveness excellent in 4 degrees C and -80 degrees C. As mentioned above, although this example was explained based on experimental data, this invention is not limited to the above-mentioned example, but various deformation is possible for it based on the meaning of this invention. For example, although experimented in the purity of 1-kestose about 99.9% of thing, it cannot be overemphasized that the thing of more low purity is also usable.

[0030]

[Effect of the Invention] Whether it uses 1-kestose independently or uses together the viable cell preservative of this invention with other viable cell preservatives, it has the preservation effectiveness excellent also in the thing [ any ] case. The viable cell preservative of this invention has effectiveness not only in cryopreservation but in preservation at the low temperature of extent which is not frozen.

[0031] Since mass production method can do 1-kestose by microorganism industry, a viable cell preservative can be offered cheaply.

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## TECHNICAL FIELD

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[Industrial Application] In case this invention saves a viable cell at a freezing condition or low temperature for a long period of time, it relates to the sperm of a required viable cell preservative especially the mammals, and fishes, and the viable cell preservative of an animal culture tissue.

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## PRIOR ART

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## EFFECT OF THE INVENTION

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## TECHNICAL PROBLEM

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## MEANS

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